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that functional cytokine-inducible as-regulatory elements are found in regions upstream of -3.8 kilobases (kb) in the 5′ flanking region of the human NOS2 gene.[9] These data show that complex mechanisms regulate NOS2 gene expression in the liver.

Although cytokines are potent inducers of NOS2 in hepatocytes, NOS2 expression appears to be differen-

tially regulated from the acute phase response, another hepatocyte gene program that is cytokine dependent.[4] Recently we demonstrated that induction of heat shock response (HSR), another common phenotype expressed in the liver during stress conditions, inhibits cytokine-induced NOS2 expression and NO synthesis in rat hepatocytes (unpublished data). The HSR is a highly conserved cellular defensive program that is expressed in response to numerous environmental stresses.[10] Induction of the HSR by toxic insults such as hyperthermia, heavy metals, or ischemia-reperfusion leads to the synthesis of various heat shock proteins, some of which are thought to confer protection to injured cells by guiding the folding and trafficking of damaged proteins. In this study we sought to determine the molecular mechanism(s) underlying the inhibition of cytokine-induced NOS2 by the HSR using a human liver cell line (AKN-1).

MATERIAL AND METHODS

Cell culture. The AKN-1 liver cell line was originally isolated from sections of normal human liver by serial dilutional cloning. These cells express high levels of endogenous NOS2 mRNA and protein in response to cytokines.[9] AKN-1 cells were plated onto 100 mm Petri dishes (Corning Co., Corning, N.Y.) and maintained at 37° C, 95% air, and 5% CO_2 in modified HCD medium.[9] Experiments were carried out when cells were 75% to 100% confluent. To induce the HSR, AKN-1 cells were either treated with sodium arsenite (Ars, 20 μmol/L) (Sigma Chemical Company, St. Louis, Mo.) or placed at 43° C for 2 hours. A cytokine mixture (CM) of human recombinant TNF-α (1000 units/ml) (R&D Systems, Minneapolis, Minn.) plus human recombinant IL-1β (100 units/ml) (gift of Craig Reynolds, National Cancer Institute) plus human recombinant IFN-γ (250 units/ml) (R&D Systems) was then added to the cultures to stimulate NOS2 expression. Ars was removed after 6 hours of incubation time by washing the cells; cells previously exposed to cytokines were maintained in their presence. Northern blot analysis for NOS2 was performed 4 hours after CM stimulation, and Western blot analysis for hsp70 was performed 18 hours after initial induction of the HSR. AKN-1 cells transduced with either a retroviral expression vector encoding the human hepatocyte NOS2 cDNA and a neomycin resistance gene (DFG-NOS2) or a retroviral expression vector containing the β-galactosidase and neomycin genes (Bag-LacZ)[11] were maintained in the presence of N^G-monomethyl-L-arginine (0.5 mmol/L) before experiments. Crystal violet staining was used to assess cell viability at the end of each study.

Nitrite plus nitrate assay. An automated procedure based on the Griess reaction[7] was used to measure 24-hour accumulation of the end products of NO, nitrite plus nitrate ($NO_2^- + NO_3^-$), from the culture medium.

cDNA probes. A 2.3 kb *Bam*HI fragment of the human hepatocyte NOS2 cDNA previously cloned in our laboratory[12] was used for Northern blot analysis. To control for the amount of RNA in each lane, Northern blot membranes were stripped with boiling 5 mmol/L ethylenediamine tetraacetic acid, 0.1% sodium dodecylsulfate, and rehybridized with a probe for 18s ribosomal RNA.

Preparation of cell lysates. AKN-1 cell lysates were collected by lysing cells in ice-cold buffer containing 20 mmol/L TES (pH 7.4), 2 mmol/L dithiothreitol, 10% glycerol, 50 μmol/L phenanthroline (Aldrich, Milwaukee, Wis.) antipain, aprotinin, leupeptin, chymostatin, pepstatin, 100 μmol/L phenylmethylsulfonyl fluoride (all reagents from Sigma Chemical Company) and subjecting the cell suspension to four cycles of freeze-thaw followed by brief centrifugation.[11] Protein concentration was measured with bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, Ill.).

Northern and Western blot analyses. RNA extraction, Northern blot analysis, and autoradiography were performed as described previously.[7] Relative mRNA levels were quantitated by PhosphorImager scanning with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, Calif.). For Western immunoblots 50 μg cytosolic proteins was separated by 8% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). The membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline solution (PBS)–Tween, washed in PBS-Tween, and then incubated for 1 hour with a monoclonal mouse anti-hsp70 antibody (1:2500 dilution; Stressgen, Victoria, B.C., Canada). The secondary antibody used was a peroxidase-conjugated goat anti-mouse immunoglobulin G in a 1:1000 dilution (Schleicher & Schuell). After washes with PBS-Tween, membranes were developed with the enhanced chemiluminescence detection system (DuPont-New England Nuclear, Boston, Mass.) and exposed to film.

Promoter analysis. Transient transfections of a 7.0 kb NOS2 promoter-luciferase construct (pNOS2(7.0)Luc) into AKN-1 cells were carried out by using Lipofectamine (Gibco BRL, Grand Island, N.Y.) as described previously.[9] After an overnight recovery from the transfection, AKN-1 cells were treated with Ars (20 μmol/L) or exposed to 43° C for 2 hours before the addition of CM. Luciferase assays (Promega, Madison, Wis.) were performed as previously described,[9] and luciferase activity was normalized to protein content. Results are reported as fold increase of luciferase activity of CM-treated cells over control cells.

Statistical analysis. Results are reported as mean ± SE. The significance of differences was determined by

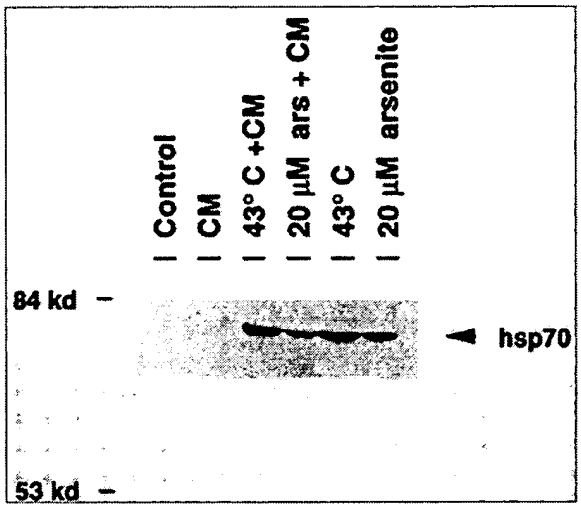

Fig. 1. Induction of HSR in AKN-1 cells. These experiments were performed twice with similar results.

ANOVA with the Statview statistics program (Abacus Concepts, Inc., Berkeley, Calif.). Statistical significance was established at a p value <0.01.

RESULTS

HSR inhibits NOS2 expression in AKN-1 cells. Stimulation of the HSR in primary hepatocytes is well characterized. However, our current studies required that we use a human liver cell line (AKN-1) expressing high endogenous NOS2 activity. To show induction of the HSR, AKN-1 cells were exposed to Ars (20 μmol/L) or hyperthermia (43° C), both of which are known to induce the HSR.[10] As shown in Fig. 1, Ars or hyperthermia induced the synthesis of hsp70 protein in AKN-1 cells. Control or CM-treated cells did not have any detectable hsp70 protein, and cytokines did not affect hsp70 protein. To determine the effect of the HSR on cytokine-induced NO synthesis, AKN-1 cells were treated with CM after pretreatment with Ars or heat. The addition of CM alone to AKN-1 cells resulted in marked expression of NOS2 mRNA and the production of large amounts of NO (13.7 nmol NO_2^- + NO_3^-/mg protein/24 hr) (Fig. 2). However, prior exposure to Ars or hyperthermia inhibited CM-induced NOS2 mRNA levels by ~86% (by PhosphorImager scanning, not shown) and NO_2^- + NO_3^- accumulation by ~60%. These results are consistent with our previous findings showing the inhibition of cytokine-stimulated NOS2 mRNA and NO production by the HSR in rat hepatocytes (unpublished data). Cell viability was greater than 70% (not shown).

HSR down-regulates cytokine-induced NOS2 promoter activity. We have recently shown that cytokines transcriptionally activate the NOS2 gene in AKN-1 cells and have demonstrated that at least 5.8 kb of the 5′ flanking region of the gene is needed to confer cytokine inducibility to the human NOS2 promoter.[9] To determine whether the HSR affected NOS2 gene transcription, we examined the effect of Ars and hyperthermia on NOS2 promoter activity. AKN-1 cells were transiently transfected with a 7.0 kb NOS2 promoter construct ligated upstream of the luciferase reporter gene and stimulated with CM. An approximately fivefold increase in luciferase activity was obtained; however, Ars or hyperthermia significantly reduced this activity by 44% and 77%, respectively, indicating that NOS2 promoter activation was being down-regulated. Ars treatment or hyperthermia did not stimulate NOS2 promoter activity (not shown). These data suggest that the HSR inhibits cytokine-induced NOS2 expression in AKN-1 cells by blocking NOS2 gene transcription.

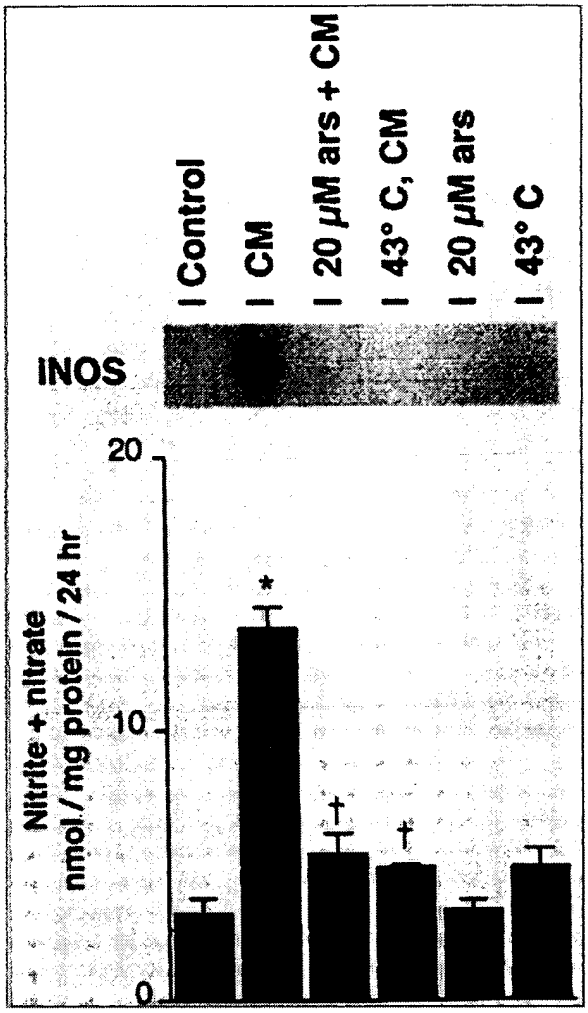

Fig. 2. HSR inhibits cytokine-inducible nitric oxide synthesis. Shown are data representative of three separate experiments. *p < 0.01 versus control; †p < 0.01 versus CM by ANOVA.

NOS2 enzyme activity is not affected by the HSR. To determine whether the HSR had any direct effects on the translation of NOS2 protein, NO production was

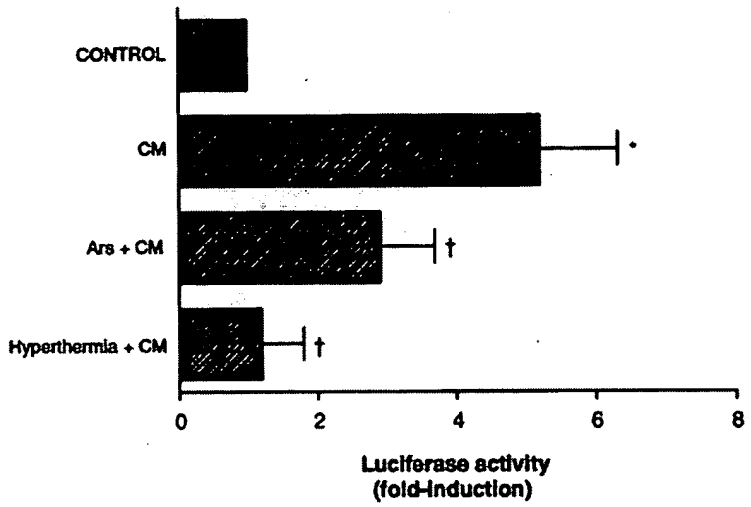

Fig. 3. HSR suppresses NOS2 promoter activity. $n = 4$. $*p < 0.01$ versus control; $\dagger p < 0.01$ versus CM by ANOVA.

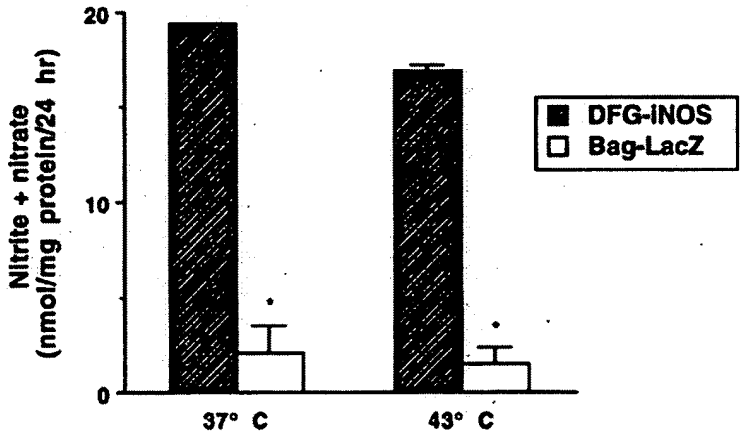

Fig. 4. HSR exerts no apparent effects on NOS2 enzyme activity. $*p < 0.01$ versus Bag-LacZ by ANOVA.

measured at 37° C and 43° C in AKN-1 cells stably expressing a retroviral-driven NOS2 cDNA (DFG-NOS2). DFG-NOS2 AKN-1 cells accumulated similar amounts of $NO_{2-} + NO_{3-}$ in the culture supernatant at both temperatures (19.4 nmol/mg protein/24 hr at 37° C and 17.0 nmol/mg protein/24 hr at 43° C), whereas control Bag-Lacz AKN-1 cells only had basal $NO_{2-} + NO_{3-}$ release. These findings show that the HSR exerts no apparent effects on NOS2 protein translation or activity.

DISCUSSION

In this study we investigated the mechanisms involved in the inhibition of cytokine-induced NO synthesis by the HSR in the AKN-1 human liver cell line. All cells, from prokaryotes to eukaryotes, are capable of expressing the heat shock phenotype in response to various toxic insults.[10] The induction of the HSR results in a profound reprogramming of cellular gene expression and leads to the production of heat shock proteins, the most inducible of which is hsp70. Fig. 1 shows that Ars or hyperthermia potently induced the synthesis of hsp70 in AKN-1 cells, indicating activation of the HSR, whereas cytokines had no apparent effect on hsp70 protein levels.

The addition of TNF-α + IL-1β + IFN-γ (CM) to AKN-1 cells stimulated high levels of NOS2 mRNA and 24-hour $NO_{2-} + NO_{3-}$ release into the medium (Fig. 2). However, prior induction of the HSR dramatically prevented NOS2 expression and enzyme activity. To determine whether NOS2 gene transcription was being affected, we investigated the effects of the HSR on NOS2 promoter induction. CM-induced promoter activity of AKN-1 cells transfected with a 7.0 kb NOS2 promoter luciferase construct was significantly decreased by Ars or

hyperthermia, implying that the HSR was blocking NOS2 expression at the level of transcription (Fig. 3). Hyperthermia caused a more potent down-regulation of NOS2 promoter activity compared with Ars treatment and may have been due to a different degree of potency between the two inducers. A recent report showed that IL-1β–induced NOS2 expression in rat pulmonary artery smooth muscle cells is also inhibited by the HSR.[13] However, transcriptional inhibition did not appear to occur. Taken together, these data indicate that NOS2 is regulated by mechanisms that are both cell and species specific. Other studies have shown that the HSR also inhibits TNF and IL-1 release in macrophages[14] and acute phase reactant synthesis in the liver.[15] Nevertheless, induction of the HSR does not lead to the inhibition of all programs of gene expression as shown in fibroblasts in which prostaglandin synthesis is enhanced with heat shock.[16]

A recent study has shown that the transcription factor NF-κB is crucial for the activation of the murine NOS2 promoter by LPS in macrophages.[17] We have also shown that NF-κB is required for the cytokine induction of NOS2 mRNA in rat hepatocytes.[18] Our preliminary data indicate that the HSR partially prevents the binding of NF-κB in electromobility shift assays of AKN-1 nuclear extracts (unpublished observations), further providing a basis for the inhibition of NOS2 promoter activity by the HSR. Interestingly, overexpression of hsp70 in murine fibroblasts does not affect NF-κB binding activity, although IL-1 and IL-6 release are inhibited.[19] In that study it was postulated that posttranslational mechanisms were involved in the inhibition of IL-6 by hsp70. Our studies with the transduced AKN-1 cells revealed that NOS2 enzyme activity was not being affected by the HSR (Fig. 4), indicating a lack of translational or posttranslational effects. We wish to emphasize that we have not directly examined a role for hsp70 in the inhibition of cytokine inducible NOS. Obviously numerous heat shock proteins and any or a number of these peptides may be involved in NOS2 inhibition.

These findings indicate that the HSR inhibits cytokine activation of the human NOS2 promoter, suggesting that NOS2 gene transcription is suppressed. Whether HSR inhibition of NOS2 expression in vivo is beneficial or adverse is unknown at this time. Overproduction of NO can lead to detrimental effects, and studies have shown that inhibition of NO decreases hepatic injury in animal models of sepsis and ischemia-reperfusion.[3, 20] Prior induction of the HSR with hyperthermia in LPS-treated rats results in decreased liver injury,[21] but whether this is due to attenuated NO synthesis is not known. One report has shown that heat shock protected rat pancreatic islet cells against exogenous NO toxicity.[22] On the other hand, attenuating NO production may have adverse sequelae because of the loss of the protective actions of NO. Furthermore, although the HSR is perceived by most to be a protective phenotype, others have shown that induction of the HSR may lead to untoward effects. Endothelial cells treated with LPS followed by heat shock or the administration of Ars underwent apoptosis, and this was attributed to the preemption of the expression of important genes necessary for promoting homeostasis such as the acute phase response.[23] Because expression of the HSR would be expected in the liver in critically ill surgical patients suffering shock, trauma, and ischemia-reperfusion, it is possible that a lack of a capacity to express NOS2 with a subsequent septic insult would lead to greater injury. Further investigation looking into the consequences of attenuated NO synthesis during the HSR is required to address these points.

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DISCUSSION

Dr. Ori D. Rotstein (Toronto, Ontario, Canada). These studies have shown us quite clearly that there is an association between the induction of heat shock protein and the alteration in the transcription of the iNOS gene. Can you bring us up to date on the causal relationship between the presence of increased heat shock protein and its effects on NF-κB and its failure to translocate to the nucleus?

Dr. de Vera. As I mentioned previously, we have shown that cytokine-inducible promoter activity is present only in regions between -3.8 kb to -7 kb in the human iNOS promoter. This is really unique in that eukaryotic genes often have inducible promoter elements found within the first 1 kb of the 5′ regulatory region. Therefore in this respect the human iNOS promoter is different.

We have shown that NF-κB plays an important role in the induction of the human iNOS gene. There is a putative NF-κB DNA binding element that is present in the proximal regions of the human iNOS promoter (around position -100) that we think is crucial for its activation. Because we have not been able to show any cytokine-inducible promoter activity in this region, the question arises on how this proximal NF-κB element can play such an important role. Our hypothesis is that the proximal region of the human iNOS promoter is necessary but not sufficient for the activation of the promoter and that this proximal NF-κB site has an important function.

Our findings in this study, namely, that the HSR inhibits NF-κB activation, have not been shown before and may offer insights into the mechanisms behind the inhibition of other genes such as the acute phase response or cytokine genes by the HSR. The exact mechanism as to how the HSR inhibits the DNA-binding activity of NF-κB is unknown at this time. It is possible that NF-κB may be prevented from translocating to the nucleus or, alternatively, heat shock factor may potentially be preventing NF-κB from binding to its target sequence.

Dr. Bauer E. Sumpio (New Haven, Conn.). I have three brief questions. First, the standard way of determining whether there is transcriptional regulation is to do nuclear run-on studies or actinomycin C inhibition studies. Would you comment on whether you have done these studies to nail down whether this phenomenon is indeed transcriptional regulated?

Second, is this finding specific for iNOS or are there other genes that are up-regulated?

The third question is along the lines that Dr. Rotstein mentioned. Have you done any DNase I protection assay "footprinting" to actually show that binding NF-κB or other transcription factors that occur are indeed altered in the presence of Ars?

Dr. de Vera. I would like to address the first and last questions together. We have not yet done DNase protection assays. We are carrying out deletional analyses of the proximal portion of the iNOS promoter and are currently in the process of mutating the NF-κB site that I have mentioned previously to determine its functionality. We do plan to perform DNase protection assays in the more upstream regions of the human iNOS promoter, but we have yet to do those studies.

We did not perform nuclear run-on assays in these studies. I agree with you that nuclear run-on transcription assays are the gold standard of showing whether there are changes in transcriptional rates of a gene. We have done nuclear run-on assays in AKN-1 cells and obtained an approximately fivefold increase in iNOS transcription rates with our CM. This fivefold increase is similar to what we obtain in our transfection assays with the 7 kb iNOS promoter construct. Therefore we believe that our transfection data are valid. Although these studies are indirect, they are certainly strongly suggestive that iNOS gene transcription is being inhibited by the HSR. Other genes such as acute phase or cytokine genes are also inhibited by the HSR, and the mechanisms involved in the inhibition of these genes are relatively unknown. Hopefully these studies will yield further information about these mechanisms.